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14. ABSTRACT In order for prostate cancer to metastasize, it must invade through a laminin-511 rich barrier. We have previously shown that the matrix metalloprotease, MT1-MMP, which is expressed in prostate cancer but not in normal prostate tissue, cleaves the laminin alpha-5 chain into four distinct fragments. This cleavage allows for increased prostate cancer cell migration in vitro. Laminin-511 cleavage also occurs in vivo in human prostate tissue. Cleavage of laminin-511 and release of laminin-511 fragments leads to altered cell function leading to increased cell migration and invasion in in vitro assays. We have demonstrated that prostate cancer cells treated with laminin-511 that has been cleaved by MT1-MMP have increased EGFR phosphorylation compared with cells grown on tissue culture plastic or intact laminin-511 in a Western blot. We have purified a recombinant 45kDa laminin-511 N-terminal cleavage fragment, which contains laminin EGF-like domains. Treatment of prostate cancer cells with soluble recombinant fragment demonstrates that the cleaved laminin fragment acts as a matrikine, activating the EGFR on prostate cancer cells in a Western blot. This work demonstrates that increased MT1-MMP expression in prostate cancer not only cleaves the major laminin surrounding prostate cancer to clear a path for migration, but also releases active fragments from the laminin-511 that signal for increased migration.

15. SUBJECT TERMS
Prostate cancer, laminin-10, MT1-MMP

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Introduction

Prostate cancer is currently the most commonly diagnosed neoplasm and the second leading cause of cancer deaths in men in the United States. Due to the aging population, prostate cancer incidence and mortality is expected to increase. Thus, improvements in prevention, early dignoses, and treatment are needed [1]. Hopefully, understanding molecular events of prostate cancer progression will lead to the development of novel therapies for the disease. A prostate cancer's ability to develop an invasive and metastatic phenotype is arguably the most important determinant in the clinical relevance of prostate cancer; and therefore, the study of factors involved in the development of this phenotype is of primary importance. There are at least two recognized phenotypes of prostate cancer. One is a clinically aggressive cancer that can become life-threatening relatively fast, while the other is a slow- growing, "latent" form, which may ever present itself clinically [2].

To date, there is no method to differentiate between these two forms of prostate cancer, leading to potential over- and under- treatment of this disease [3]. For this reason, identification and understanding of molecular alterations during prostate cancer progression is essential to distinguish between cases that will progress rapidly to advanced metastatic cancer and those with little likelihood of progressing. This will hopefully lead to more accurate prognoses and appropriate treatments. In addition, identification of biomarkers will be useful for recognizing individuals with a higher risk for prostate cancer and will allow for earlier detection and better treatment of advanced disease. In particular, there is a growing need for biomarkers that access the tumor microenvironment as predictors of metastases [4].

In order for a cancer to metastasize, it must first invade through the basement membrane that surrounds it, migrate through the stroma, invade blood vessels and travel through the bloodstream to a new location where it extravasates the vessel and begins growing at the new site. The mechanisms by which a cancer is enabled to invade and metastasize are currently under intense study. Interactions of the cell with its environment are thought to play a major role in signaling for these invasive processes to occur. Upregulation of proteolytic enzymes, such as the matrix metalloproteases, is suspected of being involved in the metastatic process. Remodeling of the extracellular matrix (ECM) through proteolysis of ECM proteins is an important step in the metastatic progression of cancer, allowing for invasion of neoplastic cells through the basal lamina (BL) and into the stroma [5]. Proteolysis creates paths for migration, releases signaling molecules such as growth factors bound in the ECM, and generates biologically active ECM fragments [6-10].

Prostate cancer is surrounded by a BL composed mainly of laminin-10, laminin-2, type IV collagen, and entactin [11]. In order for prostate cancer to invade the stroma and intravasate into the vasculature, it must move through this Ln-10 rich BL either by proteolysis or ameboid movement [12]. Ln-10 ($\alpha5\beta1\gamma1$) has been previously identified as a substrate for cell migration and cell adhesion [13]. It is now known that MT1-MMP can cleave the laminin-10 $\alpha5$ chain and this cleavage allows for increased prostate cancer cell migration. We have preliminary evidence that the laminin-10 $\alpha5$ chain protein cannot be detected in perineural invasive prostate cancer by immunohistochemistry (Figure 3). This apparent loss of laminin-10 protein could be due to complete cleavage by MT1-MMP so that antibodies to the $\alpha5$ chain cannot bind or it could be due to loss of laminin-10 expression in invasive cancer. We propose that modifications in

extracellular matrix proteins surrounding prostate cancer play an important role in the progression of the disease and an understanding in the global changes in cancer that take place as a result of these modifications will be important for future prostate cancer research, treatment, and diagnosis.

Body

Previously, we have demonstrated that the matrix metalloprotease, MT1-MMP, cleaves the laminin-10 alpha 5 chain into four distinct fragments with apparent molecular weights of 45, 160, 190, and 310 kDa. When human prostate cancer cells are plated on cleaved laminin-10, there is an increase in migration on and invasion through the laminin-10 substrate. This work was published in the journal *Neoplasia* in April 2005. We are currently in the process of examining the biologic effects of these fragments, and the potential cleavage of laminin-10 in human prostate cancer.

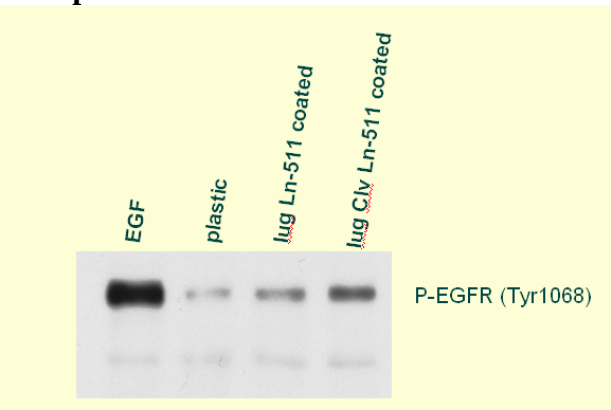
To test the biologic effects of the laminin-10 cleavage fragments, recombinant proteins corresponding to laminin alpha 5 chain cleavage products are being produced. To date, the 45kDa N-terminal end of the alpha 5 chain has been expressed in E.Coli and purified on a Ni^{2+} column. This protein has been tested to determine whether it is the cause of the biologic effects observed when prostate cancer cells are treated with cleaved laminin-10 (EGFR phosphorylation, proliferation, migration). We have treated DU-145 cells with purified laminin-10 and purified laminin-10 that was treated (cleaved) with MT1-MMP. These experiments demonstrated an increase in EGFR phosphorylation at tyrosine 1068. Purified laminin-10 induced some EGFR activation, but cleaved laminin-10 demonstrated a more robust activation. We have found that treating DU-145 prostate cancer cells with 1μM concentration of the 45kDa fragment induces phosphorylation of the EGF receptor at tyrosine 1068, similarly to treating DU-145 cells with 1μg purified, cleaved laminin-10.

We also have evidence that the extracellular matrix protein, laminin-10, though present in normal prostate and prostate cancer, may be downregulated in perineurally invasive prostate cancer. With current techniques, we do not know whether this apparent lack of protein expression by immunohistochemistry with a laminin-10 alpha 5 chain antibody is due to loss of protein, or clipping of the alpha 5 chain by increased MT1-MMP expression. Since there are currently no antibodies available to the cleaved forms of the alpha 5 chain, we chose to examine mRNA expression of the protein to determine if it is being expressed in perineurally invasive prostate cancer. RNA probes were generated against the human laminin alpha 5 chain and human MT1-MMP. These probes are currently in use using *in situ* hybridization to detect message for these proteins in human prostate cancer tissue. The technique for *in situ* hybridization on human prostate tissues has been worked out, and preliminary experiments with the alpha 5 chain probe indicate that mRNA is present in prostate cancer, though to a lesser extent than mRNA present in normal glands. Perineurally invasive prostate tissue still needs to be examined.

Hypothesis: LM-511 cleavage by MT1-MMP results in decreased adhesion and increased migration of prostate cancer cells due to release of biologically active LM-α5 fragments

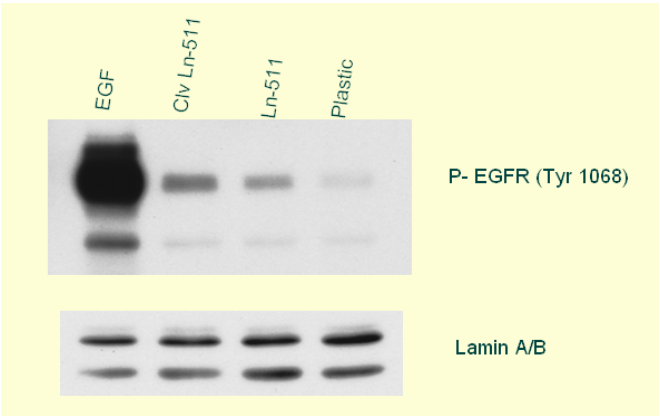
containing EGF-like domains that function as matrikines, can bind and activate the EGF receptor.

Activation of EGFR on cells plated on cleaved LM-511.



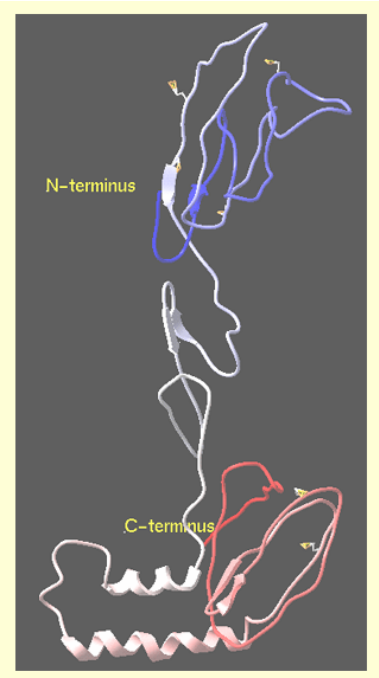
LM-511 (μg) was coated on a 6-well tissue culture plate and treated with catalytic domain of MT1-MMP (2.1 nmol) or left intact. DU-145 cells were then plated on uncoated and LM-511 coated wells. EGF was added to an uncoated well as a control for activation. 16 hours after plating, cells were lysed and analyzed for EGFR activation at Tyr 1068. Results demonstrate EGFR activation when cells were plated on intact LM-511, with an increase in EGFR activation when cells were plated on cleaved LM-511.

Activation of EGFR with soluble LM-511 fragments.



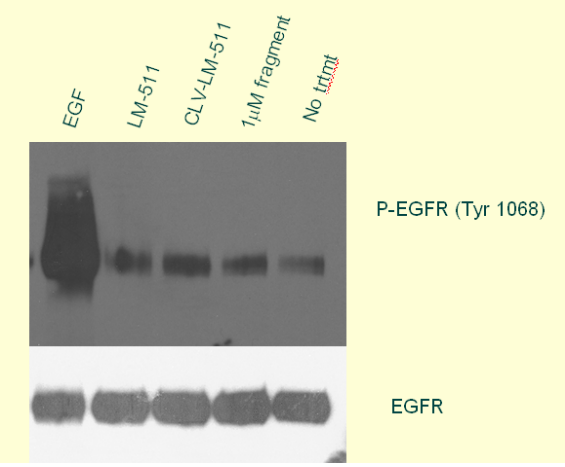
DU-145 cells were seeded in a 6-well tissue culture plate and stimulated with: EGF, LM-511 fragments produced by incubating intact LM-511 with catalytic domain of MT1-MMP 16 hr, intact LM-511, or were left untreated. Results demonstrate that cleaved LM-511 fragments activate EGFR to a greater extent than intact LM-511, and that the receptor is not activated with no treatment. Lamin A/B protein was used as a loading control.

Structural representation of LM-α5 chain 45 KD fragment.



The LM-α5 chain 45 kDa fragment we have produced in E.Coli has been modeled based on its amino acid sequence. The fragment contains a complete EGF-like domain at its C-terminus.

LM-α5 45 KD fragment activates EGFR.



DU-145 cells were treated with EGF, intact soluble LM-511, cleaved soluble LM-511, 45 kDa soluble fragment, or left untreated. Results indicate that the 45 KD fragment activates EGFR above endogenous levels (untreated). Total EGFR protein is shown as a loading control.

Key Research Accomplishments

1. Trained in recombinant protein design, synthesis, and purification.
2. Trained in in situ hybridization technique.
3. 2005, NCI-sponsored Tumor Microenvironment Training Program: Organotypic Models Training Program in Dr. Isaiah Fidler's laboratory in Houston, TX participant. .
4. Acquired knowledge novel migration and invasion assays. Recombinant 45kDa fragment of laminin alpha5 chain was made and purified.
5. Biochemical assays of EGFR phosphorylation were performed using phospho-specific antibodies.
    a. EGFR was phosphorylated in when treated with or grown on purified, cleaved laminin-10
    b. EGFR was phosphorylateed after treatment with purified 45KD recombinant laminin alpha5 chain fragment.

Reportable Outcomes

Bair EL, Bowden, GT, Nagle, RB "Cleaved Laminin-α5β1γ1 Fragment Activates EGFR in Prostate Cancer Cells" An AACR Special Conference in Cancer Research "Cancer, Proteases, and the Tumor Microenvironment" November 30-December 4, 2005**,** Bonita Springs, Florida.

Conclusions

- LM-511 cleaved with MT1-MMP contains potentially bio-active fragments.
- Cleaved LM-511 fragments activate the EGFR at Tyr 1068.
- Recombinant 45 kDa LM-α5 chain demonstrates activity in activating the EGFR.

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2004-2006, University of Arizona, Cancer Center, Postdoctoral Research Associate
2000-2004, Graduate Research Assistant, University of Arizona, Cancer Biology, Arizona Cancer Center
1999-2000, Research Assistant, University of Arizona, Radiation Oncology, Arizona Cancer Center
1999-1999, Undergraduate Biology Research Program, University of Arizona, Radiation Oncology, Arizona Cancer Center
1998-1998, Undergraduate Biology Research Program, University of Arizona, Radiation Oncology, Arizona Cancer Center

Funding Received:
- DOD Postdoctoral Traineeship Award, "Effects of Modifications in the Laminin-10 Basal Lamina on Prostate Cancer Invasion", $, from 10/2005 to 10/2007
- Cancer Biology Postdoctoral T-32 Training Grant, National Institutes of Health, University of Arizona, from 6/2005 to 10/2005
- Cancer Biology Predoctoral T-32 Training Grant, National Institutes of Health (NIH), University of Arizona, from 8/2002 to 12/2004

Publications:
- **Bair EL**, Nagle RB. Changing Extracellular Matrix Ligands During Metastasis. In Cress AE and Nagle RB (eds), Cell Adhesion and Cytoskeletal Molecules in Metastasis, Springer Science, Submitted.
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Honors and Awards Information:

- 2005, NCI-sponsored Tumor Microenvironment Training Program: Organotypic Models Training Program in Dr. Isaiah Fidler's laboratory in Houston, TX participant.
- 2005, Young Pathologist Fellowship, American Society for Investigative Pathology (ASIP), University of Arizona
- 2003, Annual Meeting Predoctoral Travel Award, American Society for Cell Biology (ASCB), University of Arizona
- 2000, John and Betty Anderson Memorial Scholarship Endowment, University of Arizona
- 1999, Departmental Honors, Biochemistry/ Molecular Biology, Wittenberg University
- 1997, Saturn/NationsBank Undergraduate Fellowship
- 1996-2000, Wittenberg University Scholar Undergraduate Fellowship

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Education

Degree/Diploma	Institution	Date
Post Doctoral Fellow	Arizona Cancer Center, University of Arizona, Tucson, AZ, U.S.A.	June 2006-Present
Ph.D. (Molecular and Cellular Biology) Supervisor: Dr. Anne E. Cress	Arizona Cancer Center, University of Arizona, Tucson, AZ, U.S.A.	August 2001-May 2006
M.Sc. (Microbiology)	Bhavans College, Mumbai University, Mumbai, India	June 1999 – May 2001
Diploma in Clinical Analysis	Sophia College, Mumbai, India.	June 1993 – May 1994
B.Sc. (Microbiology)	Sophia College, Mumbai University, Mumbai, India.	June 1990 – May 1993

Teaching Experience

2005: Research supervisor for Tiama Friend undergraduate Minority Health Disparities Summer Research Program, University of Arizona, AZ.
2005: Mentored following students in their research projects: Janice Lopez, Win Win, Kelvin Pond and Sabrina Sykes.
2003: Teaching Assistant for BIOC 411 (Molecular Biology).
2002: Teaching Assistant for MCB 410 (Cell Biology).

Technical Skills

Western Blotting, Immunoprecipitation, Immunocytochemistry, Immunohistochemistry, Flowcytometry, PCR and Cell Culture.

Honors and Awards

2006 Department of Defense Post-doctoral Fellowship Award
2004 Women in Science and Engineering Travel Award.

Poster Presentations

"Integrin α6 cleavage blocked by tetraspanin CD151 interaction"
Sangita C. Pawar, Jaime M.C. Gard, Raymond Nagle, Anne E. Cress.
Membrane Organization by Tetraspanins and Small Multi-Transmembrane Proteins.
FASEB Summer Research Conferences, Tucson, AZ.
July 2006

"Integrin α6 clipping: A novel modification to modulate cell migration" Sangita C. Pawar, Manolis C. Demetriou, Raymond B. Nagle, G. Tim Bowden and Anne E. Cress.
American Society for Cell Biology, Moscone Center, San Francisco, CA.
December 2005.

"Integrin Clipping: Enhancing osteolytic activity in prostate cancer." Sangita C. Pawar, Tamara E. King, Frank Porreca, Raymond B. Nagle, Anne E. Cress.
National Institute of Health, Bethesda, MD.
May 2005

"Integrin α6 clipping: A novel modification to modulate cell migration" Sangita C. Pawar, Manolis C. Demetriou, Michael E. Pennington, Kevin A. Kwei, Marianne B. Powell, G. Tim Bowden, and Anne E. Cress.
American Society for Matrix Biology, San Diego, California.
November 2004.

Peer Reviewed Publications

Sangita C. Pawar, Manolis C. Demetriou, Raymond B. Nagle, G. Tim Bowden and Anne E. Cress. "Integrin α6 clipping: A novel modification to modulate cell migration." *Exp. Cell Res. 2007 Jan 17*

Oshiro MM, Kim CJ, Wozniak RJ, Junk DJ, Munoz-Rodriguez JL, Burr JA, Fitzgerald M, **Pawar SC**, Cress AE, Domann FE, Futscher BW. (2005). Epigenetic silencing of DSC3 is a common event in human breast cancer. *Breast Cancer Res. 2005;7(5):R669-80.*

Abstract Published
Sangita Pawar, Tamara King, Frank Porreca, Ray Nagle, Anne Cress
Integrin clipping: Enhancing osteolytic activity in prostate cancer. *Journal of Bone and Mineral Research. 2005; 20 (2): P40*

Manuscript in preparation

Sangita Pawar, Tamara King, Lisa Majuta, Ray Nagle, Frank Porreca, Anne Cress

"The α6p variant of the α6 integrin plays a critical role in osteolytic activity and bone-cancer induced nociception in prostate cancer in a murine bone cancer model."

Sangita C. Pawar and Anne E. Cress. "Integrin α6 cleavage blocked by tetraspanin CD151 interaction".

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